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MORPHOLOGICAL AND BEHAVIORAL IDENTIFICATION OF THE SENSORY STRUCTURES MEDIATING PHEROMONE RECEPTION IN THE BLUE CRAB, *CALLINECTES SAPIDUS*

RICHARD A. GLEESON

*Monell Chemical Senses Center, 3500 Market St., Philadelphia, PA 19104, and *Whitney Marine Laboratory, University of Florida, Route 1, Box 121, St. Augustine, FL 32084*

Abstract

Scanning electron microscopy was used to survey the aesthetasc tuft on the outer flagellum of the antennule (1st antenna) in order to identify sensilla potentially involved in pheromone detection by the male blue crab. These studies showed that the tuft of each antennule is divided into a mesial and lateral half by a region of cuticle from which no sensilla arise. Two setal types were revealed: the aesthetascs and previously undescribed sensilla which originate exclusively on the mesial side of the tuft and project to the lateral half between the rows of aesthetascs. Experiments were performed in which the mesial half, lateral half, or entire aesthetasc tuft was bilaterally ablated from the antennules of test males. As revealed by behavioral tests, pheromone responses in "mesial half" and "lateral half" ablation groups were reduced 22% and 21%, respectively, relative to control ($P > 0.10$); whereas a highly significant ($P < 0.005$) response decrement (80% relative to control) occurred in the "entire tuft" ablated group. The data suggest that pheromone reception in the male blue crab is effected via the aesthetascs. The relationship of these findings to those for other decapod crustaceans is discussed.

Introduction

Previous work demonstrated the presence of a pheromone in the urine of pubertal females of *C. sapidus* which triggers courtship behavior in males (Gleeson, 1980). It was further shown that detection of this pheromone occurs via chemoreceptors located on the outer flagellum of the antennules (first antennae) as indicated by the lack of courtship responses for males in which the outer flagella were ablated.

In the present study the outer flagellum was examined using scanning electron microscopy (SEM) to identify structures potentially involved in pheromone reception. This effort focused on the aesthetasc tuft region since these sensilla are presumed to be chemosensory ("olfactory receptors") in decapods (Ache, in press). Based on the morphological information, various lesions were made in the tuft and any decrement in pheromone response was assessed behaviorally.

Materials and Methods

Morphology

Antennules were treated in Karnovsky's fixative for two to two and a half hours, rinsed in 0.1 *M* sodium cacodylate buffer, and dehydrated through a graded ethanol

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* Present address.

series. The samples were then transferred to acetone, subjected to critical point drying, and, after gold coating, examined with a scanning electron microscope.

To evaluate the permeability of structures on the outer flagellum, the crystal violet technique of Slifer (1960) was utilized. Antennules were fixed in a 10% solution of formalin for 24 hours, then rinsed in water and exposed to a 0.5% solution of crystal violet for periods varying from five seconds to 10 minutes. After two rinses in distilled water the specimens were dried, cleared in xylene, and mounted for inspection under the light microscope.

Ablation experiments

All studies were performed during the summer months using the facilities at the University of Maryland's Marine Products Laboratory in Crisfield, Maryland. Animals were obtained locally from commercial sources, held in tanks (1.2 × 2.4 × 0.3 m) with a flow-through water system, and sustained on a diet of fish.

A test-tank (1.0 × 1.0 × 0.2 m) in which the water depth was maintained at 10 cm via a standpipe drain was used for all behavioral testing. Water filtered to 10 μm was introduced to one corner of the tank at a rate of approximately five liters per minute. As a source of pheromone, three to six pubertal females (those within six days of undergoing their maturity molt) were retained in an acrylic cylinder (15 cm height by 30 cm diameter) which was positioned close to the inflow corner of the test-tank.

In each trial six male crabs were introduced to the test-tank immediately following placement of the females within the cylinder. The activity of the males was observed over a five minute period after which the position of the inflow delivery tube was switched to overflow the water in the cylinder. The actions of the males were then noted over a second five minute observation period and courtship responses recorded as defined previously (Gleeson, 1980). The criteria used to identify courtship behavior were:

1) A courtship display—chelae extended in the lateral position, swimming appendages (fifth pereopods) rotated anterodorsally and waved from side to side above the carapace, and walking legs (second to fourth pereopods) extended such that the body is elevated to a near maximum height above the substrate; or

2) An approach towards another test-male with chelae extended in the lateral position, followed by an attempt to cradle-carry the approached individual.

All males were pre-tested in the apparatus, and only those exhibiting courtship responses were used for ablation treatments. These treatments involved bilateral antennule operations performed under a dissecting microscope. The crabs were restrained and each antennule held in position by a clamp mounted on a micromanipulator which allowed positioning the antennule such that the aesthetasc tuft was accessible for ablation. Four treatment categories were examined using randomly selected males:

1) *Ablation of the entire aesthetasc tuft.* Water was blotted from the tuft and the hairs manipulated from their normally recumbent position to allow cutting the entire tuft with micro-dissecting scissors.
2) *Ablation of the mesial portion of the tuft.* Again, this involved blotting the tuft and manipulating the hairs from their recumbent position. Fine-tipped forceps were used to pluck all of the hairs from the mesial half of the tuft, leaving the aesthetascs of the lateral half intact.

FIGURE 1. Lateral view of antennule tip showing aesthetasc tuft (arrowhead) on outer flagellum. Inner flagellum was removed. Scale bar = 700 μm.

3) *Ablation of the lateral portion of the tuft.* The procedure was as in (2) except that the lateral half of the tuft was removed and the mesial portion left intact.
4) *Sham control.* This process was as for all of the above treatments, but with no cutting or plucking of hairs within the tuft.

Between 24 to 48 hours after ablation treatments the males' pheromone responses were tested. In order to reduce the incidence of false negatives (*e.g.*, lack of response due to the stimulus failing to reach receptor sites), each male was examined in up to three trials. Two untreated control males were simultaneously tested with treated animals in every trial, and any trial in which none of the six males responded was voided.

At the conclusion of the behavioral tests, the antennules of the treated males were removed and prepared for SEM inspection.

RESULTS

Morphology

The outer flagellum of the antennule (Fig. 1) is approximately 2 mm in length and characterized by a series of over 30 segments which give it flexibility. A prominent feature of this flagellum is the tuft of approximately 650–700 aesthetasc hairs which arise from grooves distally situated on the ventral surface of most flagellar segments (Fig. 1 and 2A). This tuft is divided into mesial and lateral halves by a central region of cuticle from which no aesthetascs arise (Fig. 3A). The aesthetasc setae are from 700–1000 μm in length and approximately 10–12 μm in diameter. Three to five distinct bulges (Fig. 3B) are characteristic of the proximal region of

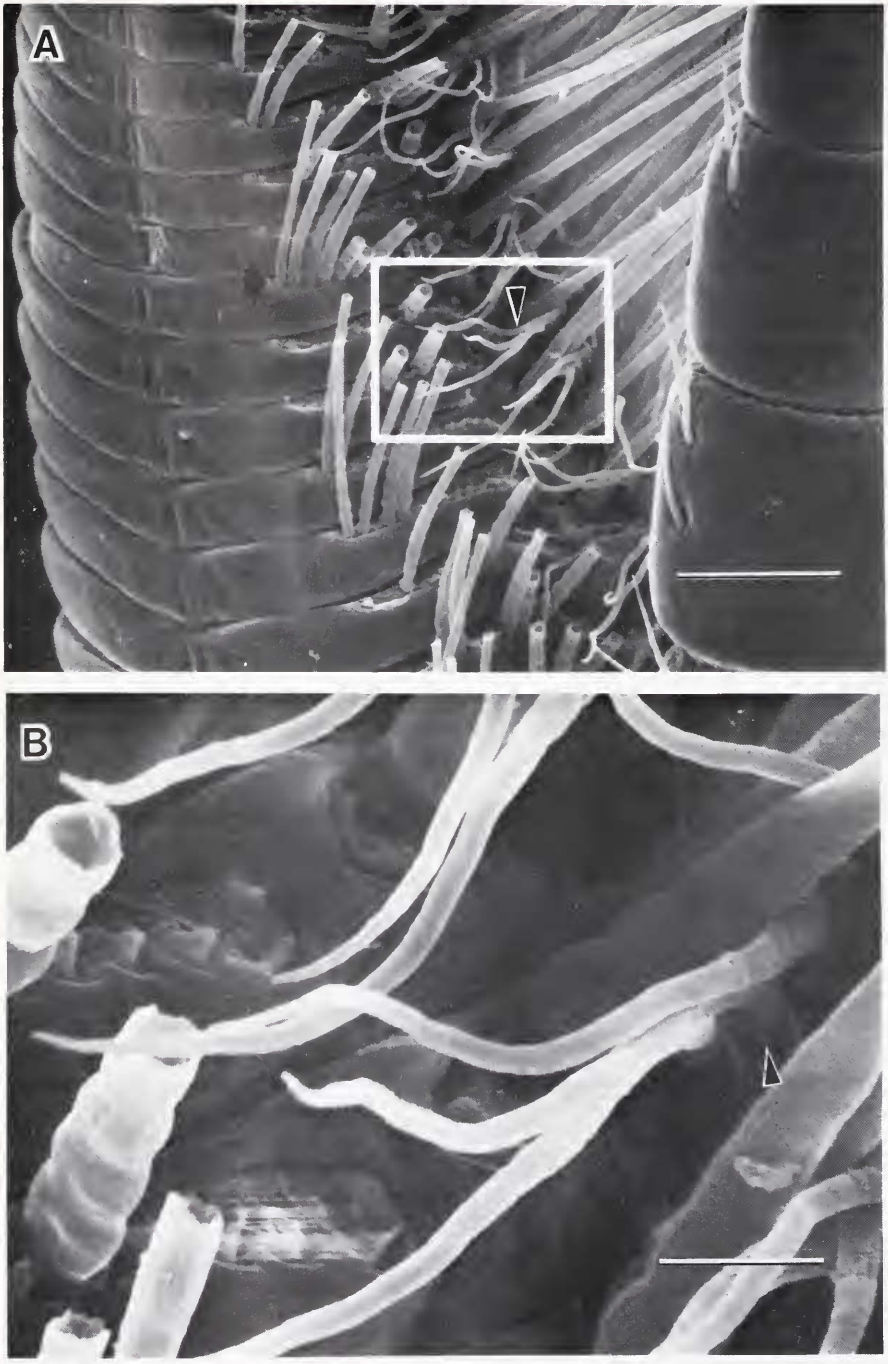

FIGURE 2. (A) Ventro-lateral view of tuft region. Lateral half of tuft was removed allowing visualization of the asymmetric sensilla (arrowhead). Aesthetascs (broken) in groove on the distal border of a flagellar segment are indicated by the arrow. Scale bar = 135 μm. (B) 5× magnification of the enclosed region in (A). Arrowhead indicates socket location of an asymmetric sensillum on mesial side of tuft. Scale bar = 27 μm.

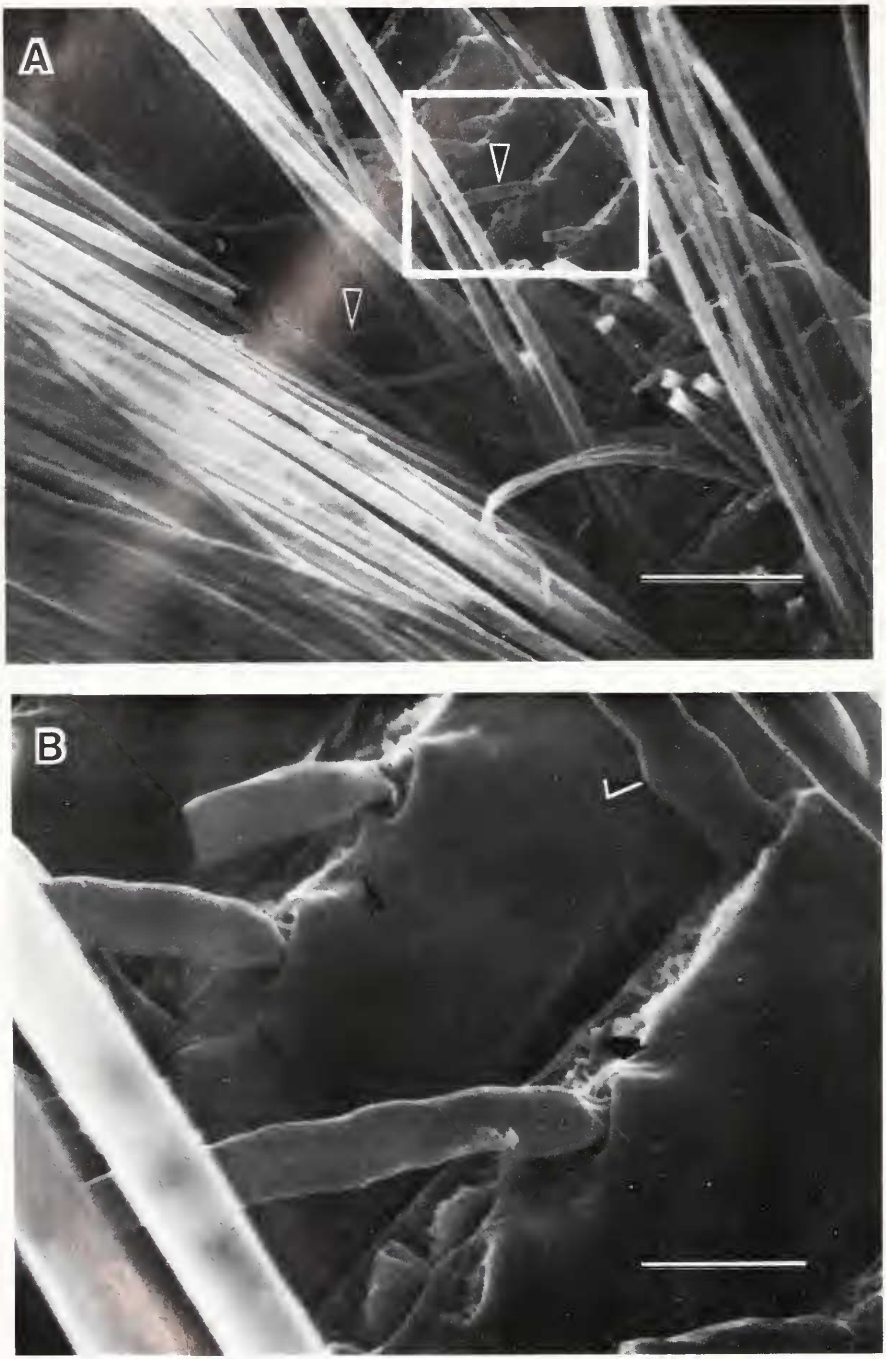

FIGURE 3. (A) Ventral view of tuft in which a portion of the mesial half was removed. Note central region of cuticle lacking sensilla (arrows). Asymmetric sensilla indicated by arrowheads. Scale bar = 100 μm. (B) 5× magnification of the enclosed region in (A). Arrow indicates socket of asymmetric sensillum. Black arrowheads show pore structures in cuticle. Annular bulge in basal region of aesthetasc indicated by black on white arrowhead. Scale bar = 20 μm.

each aesthetasc, and these give way to periodic annulations (about 30 μm apart) for the remainder of the hair shaft until the seta tapers to a tip, approximately 2 μm in diameter, which lacks a terminal pore.

Confined to the mesial half of the tuft, and proximal to the aesthetasc row of each segment, are groups of sensory hairs (0–4 per flagellar segment) with an external morphology unlike that of the aesthetascs. These setae, herein referred to as asymmetric hairs because of their exclusively mesial origin, arise from sockets which project from the cuticle at an angle such that the hairs extend across the tuft from the mesial to the lateral side (Fig. 2A, B and 3A, B). The asymmetric setae range in length from 170 to 220 μm, with diameters between 6 and 8 μm at the base, tapering gradually to a 1 μm tip with no terminal pore. Numbers of these hairs range from 46 to 70 per flagellum.

The only other surface features in the tuft region are small pores (0.3–0.6 μm in diameter) which are distributed along the distal portion of each flagellar segment just proximal to the groove from which the aesthetascs arise (Fig. 3B). Accurate counts of these structures are lacking, but the numbers range on the order of 20 to 60 per segment. Observations using light microscopy revealed that the pores are openings of canals extending 3–4 μm through the cuticle from spherical chambers (approximately 3 μm in diameter) which are situated on the inner surface of the cuticle. The aesthetasc tuft is the only region of the antennule in which these pore structures are found.

Permeability studies using crystal violet showed that both the aesthetascs and asymmetric setae were penetrated within five seconds. The asymmetric hairs were stained along their entire length, whereas a differential penetration occurred in the aesthetascs. The basal region of each aesthetasc (that section in which the annular bulges are located) was less darkly stained than the remaining portion of the hair, even after 10 minutes of exposure.

Concurrent SEM studies using antennules from females revealed no obvious sexual dimorphism in numbers or morphological types of sensory structures on the outer flagellum.

Ablation experiments

As revealed by SEM, more than 90% of the setae in the targeted regions of the aesthetasc tuft were removed or otherwise lesioned in nearly all experimental animals (Fig. 4 and 5). The results of these studies are graphically depicted in Figure 6. Removal of the mesial and lateral portions of the aesthetasc tuft produced nearly equal reductions in the response levels of males. These reductions are not statistically significant, however, when compared to the control group using a Chi-square evaluation ($P > 0.10$). In contrast, for males in which the entire tuft was ablated, the response decrement is highly significant ($P < 0.005$) when compared to any of the other treatment categories. These latter data are further supported by an additional group of seven males in which the aesthetasc tufts were similarly ablated; all were unresponsive to pheromone stimulation when examined in two trials each.

Discussion

This study provides evidence which, in conjunction with previous morphological, behavioral, and physiological work, corroborates the postulated chemosensory function of the aesthetasc setae (see for example Laverack, 1964; Ghiradella *et al.*, 1968; Hazlett, 1971). Specifically, the data establish that these setae are of critical importance to the male *C. sapidus* in detecting the pheromone of the pubertal

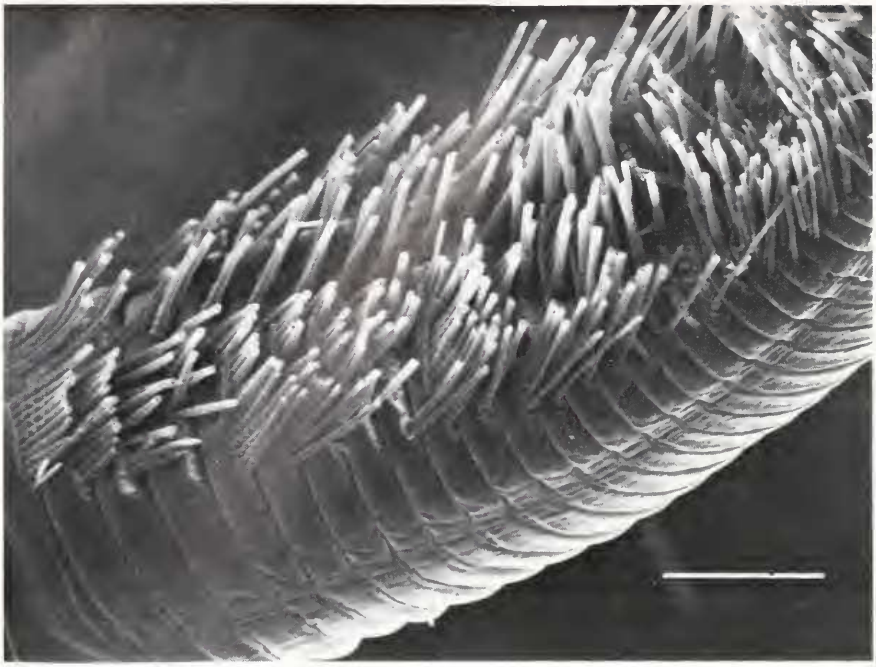

FIGURE 4. Ventro-mesial view of tuft region in which setae were cut with micro-dissecting scissors. Scale bar = 200 μm.

FIGURE. 5. Ventro-mesial view of tuft region in which mesial half was removed using forceps. Scale bar = 200 μm.

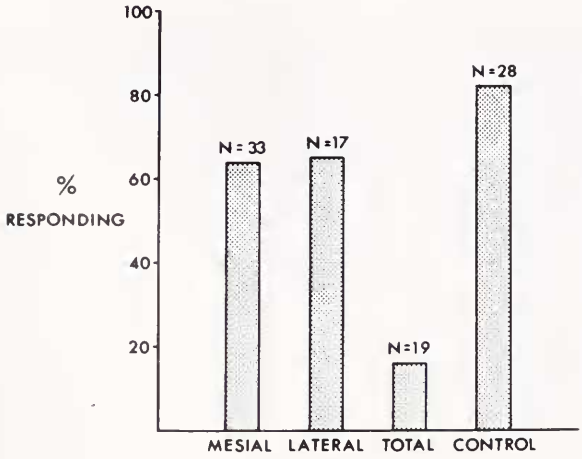

FIGURE. 6. Courtship responses of males in which the mesial half (mesial), lateral half (lateral), or entire (total) aesthetasc tuft was ablated from both antennules. Sham control (control) procedure was as for other treatment groups, but with no removal of setae. N = number of animals tested.

female, thus extending the findings of earlier experiments which localized this detection to the outer flagellum of the antennules (Gleeson, 1980).

Cutting the tuft (entire tuft ablation treatment) reduced the length of the aesthetascs such that less than one third remained intact, and this procedure decidedly blocked pheromone detection in males so treated. On the other hand, despite lesions to half the tuft, the animals in the mesial and lateral ablation groups retained their ability to detect the pheromone and also appeared equally competent in this detection capability, albeit at a reduced level relative to control. This implies that the receptors required for pheromone recognition are common to both halves of the tuft. If convergence of primary sensory neurons onto second order olfactory cells is involved in amplifying a pheromone signal (van Drongelen *et al.*, 1978), the reduced response level in these treatment groups might therefore reflect an increase in detection threshold resulting from loss of half the peripheral input to second order cells.

SEM inspection of the aesthetasc tuft region revealed relatively few sensory structures as compared, for example, to *Pagurus* (Snow, 1974) and *Panulirus* (Laverack, 1964). Other than the aesthetascs the only setae in this zone are the asymmetric hairs which are confined to the mesial half of the tuft. Their removal clearly did not alter pheromone detection in test males as indicated by the equally responsive mesial and lateral ablation groups. They are quite permeable to crystal violet, however, suggesting a possible chemoreceptive role, but this remains to be determined physiologically. Extracellular recordings from axons of cells innervating the asymmetric hairs revealed that these structures are at least mechanosensory. Deflection of the hair using a fine glass probe elicits phasic bursts of action potentials (Gleeson, unpublished data). The orientation of the asymmetric setae (*i.e.*, projection across the tuft between the rows of aesthetascs) in conjunction with this preliminary physiological data suggests that these structures may serve to monitor water flow through the tuft, such as would occur during flicking of the antennule (Schmitt and Ache, 1979).

The significance of the pores located exclusively in the tuft region is an intriguing unknown. Similar structures have been found associated with the aesthetascs of

Homarus americanus (Atema, 1977; Derby, 1982) and with certain setal types on the antennae of the sergestid shrimp, *Acetes sibogae australis* (Ball and Cowan, 1977). However, since data on the underlying structure of these pores is incomplete, speculation as to their function must await further study.

The question of pheromone receptor location has been addressed at various levels in other crustaceans. For *Palaemon paucidens*, Kamiguchi (1972) reported that the inner branch of the bifurcated outer flagellum of the antennule is relatively longer in males than in females and has a greater number of sensory (presumably aesthetasc) hairs. Although no experiments were conducted to test the hypothesis, it is postulated that this dimorphism is related to sex pheromone detection on the part of the male as has been found to be the case in many insect species (Schneider, 1964). A similar sexual dimorphism in the quantitative distribution of aesthetascs has been reported for several other crustacean groups as well (Barber, 1961).

Christofferson (1970) noted that removal of the aesthetasc-bearing outer flagellum [erroneously labeled the inner flagellum in that study and uncorrected in Dunham's (1978) review] from the antennules of the male *Portunus sanguinolentus* blocked the behavioral response to the female's sex pheromone. This response was not affected in control animals in which the inner flagellum (mislabeled the outer flagellum) was removed.

Based on ablation and electroantennulogram studies, Ameyaw-Akumfi and Hazlett (1975) and Ameyaw-Akumfi (1976) concluded that the inner (non-aesthetasc bearing) flagellum of the antennule in the male crayfish, *Procambarus clarkii*, contains chemoreceptors mediating sex recognition. The evidence on which this conclusion is based, however, is not entirely convincing. Although it is stated that test animals were unresponsive following removal of the inner flagellum, no data are presented for evaluation. Furthermore an important control condition is lacking: namely, an examination of test animals following ablation of the outer flagellum. Since the physiological data do not contribute to a resolution of this issue, the potential role of the aesthetascs in sex recognition by *P. clarkii* remains uncertain. Indeed, the situation is further confounded by the experiments of Itagaki and Thorp (1981) who used a flow-through design to examine chemical communication in *P. clarkii* and found no evidence for chemically mediated sex recognition in this species.

Dahl *et al.*, (1970a, b) present evidence suggesting pheromone reception in *Gammarus duebeni* occurs via calceoli which are male-specific sensory structures located on the second antennae. Their hypothesis is based on the apparent binding of a female-specific natural product to the calceoli, as demonstrated in males exposed to water in which radiolabeled females were retained. Recent work by Lyes (1979) has supported this hypothesis: "masking" or ablating the second antennae of the male *G. duebeni* forestalls pairing with females. However, Hartnoll and Smith (1980) found it necessary to remove both the first and second antennae to significantly block pairing; indicating that recognition of premolt females can be mediated via sensory structures other than the calceoli.

In summary, the information to date identifying structures mediating pheromone reception in decapod crustaceans is fairly limited and in some cases requires further experimentation. The present study implicates the aesthetascs as important receptors for sex pheromone detection in *C. sapidus*, but whether these structures prove to generally function in this capacity for decapods must await future comparative work specifically addressing the role of these setae in sex recognition.

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